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Overproduction of Pex5p Stimulates Import of Alcohol Oxidase and Dihydroxyacetone Synthase in a *Hansenula polymorpha pex14* Null Mutant*

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***Hansenula polymorpha* Δ*pex14* cells are affected in peroxisomal matrix protein import and lack normal peroxisomes. Instead, they contain peroxisomal membrane remnants, which harbor a very small amount of the major peroxisomal matrix enzymes alcohol oxidase (AO) and dihydroxyacetone synthase (DHAS). The bulk of these proteins is, however, mislocated in the cytosol. Here, we show that in Δ*pex14* cells overproduction of the PTS1 receptor, Pex5p, leads to enhanced import of the PTS1 proteins AO and DHAS but not of the PTS2 protein amine oxidase. The import of the PTS1 protein catalase (CAT) was not stimulated by Pex5p overproduction. The difference in import behavior of AO and CAT was not related to their PTS1, since green fluorescent protein fused to the PTS1 of either AO or CAT were both not imported in Δ*pex14* cells overproducing Pex5p. When produced in a wild type control strain, both proteins were normally imported into peroxisomes. In Δ*pex14* cells overproducing Pex5p, Pex5p had a dual location and was localized in the cytosol and bound to the outer surface of the peroxisomal membrane. Our results indicate that binding of Pex5p to the peroxisomal membrane and import of certain PTS1 proteins can proceed in the absence of Pex14p.**

Most organellar proteins in eukaryotic cells are synthesized on free or membrane-bound cytosolic ribosomes and sorted to their final destination by unique targeting signals. These signals are recognized by specific receptor proteins, which are either components of the cytosol (*e.g.* signal recognition particle for the endoplasmic reticulum (1) or importin α for nuclear proteins (2)) or are bound to the membrane of the target organelle (*e.g.* Tom proteins at the mitochondrial outer membrane (3)). Following recognition of the targeting signal and subsequent routing of the receptor-cargo complex to the actual import site, the protein is transported into the organelle. Generally, proteins are released from their receptors prior to import. However, in the case of nuclear protein import the receptor dissociates after transport through the nuclear pore complex, followed by export of the receptor back to the cytosol (3).

For peroxisomal matrix proteins comparable mechanisms may exist (4). Sorting of these proteins is mediated by specific targeting signals, termed PTS1 and PTS2. The PTS1 is located at the extreme carboxyl terminus of most peroxisomal matrix proteins and consists of the tripeptide SKL-COOH or a conserved variant thereof (5–7). In the methylotrophic yeast *Hansenula polymorpha,* the key enzymes of methanol metabolism, alcohol oxidase (AO),[1] dihydroxyacetone synthase (DHAS), and catalase (CAT), all contain a PTS1 (8, 9). The PTS2, a nonapeptide with the consensus RLX_5(H/Q)L, is located at the amino terminus and observed for a limited number of matrix proteins (7, 10, 11). Typical examples of these are peroxisomal thiolases (12, 13) and *H. polymorpha* amine oxidase (14).

The *PEX5* gene encodes the receptor for PTS1 proteins, Pex5p (15–17). Yeast *PEX5* null mutants (Δ*pex5*) are characterized by a complete block of PTS1 protein import. In such mutants the PTS2 protein import machinery is normally functioning, which indicates that both pathways can act independently in yeast. This was confirmed by the finding that in *PEX7* null mutants, which lack the PTS2 receptor Pex7p, PTS2 proteins are mislocated in the cytosol, whereas PTS1 proteins are still properly imported (18, 19).

Pex5p binds the PTS1 of newly synthesized peroxisomal matrix proteins upon their synthesis in the cytosol. Subsequently, this receptor-cargo complex is thought to interact with proteins at a putative Pex5p docking site at the peroxisomal membrane (11, 20). Peroxins proposed to be involved in the docking and subsequent translocation process include Pex13p (21–23), Pex14p (24–26), and Pex17p (27). The absence of either one of these proteins results in defects in both PTS1 and PTS2 protein import. In various genetic and biochemical studies a direct interaction of Pex5p with Pex13p and Pex14p has been demonstrated (21, 22, 25, 26, 28). Moreover, interactions between Pex13p and Pex14p and between Pex14p and Pex17p have been reported (27).

Our current working model of PTS1 protein import in *H. polymorpha* includes that in the Pex5p-cargo complex is translocated across the peroxisomal membrane into the organellar matrix, followed by dissociation and subsequent export of Pex5p from the matrix back to the cytosol (4). This model is based on the finding that in this organism Pex5p is present both inside peroxisomes and the cytosol (17). Also, evidence has been obtained that Pex4p, a ubiquitin-conjugating enzyme, plays a crucial role in recycling of Pex5p back to the cytosol (29). This was evident from the finding that overexpression of *PEX5* suppresses the PTS1 protein import defect in *H. poly-*

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[1] The abbreviations used are: AO, alcohol oxidase; DHAS, dihydroxyacetone synthase; CAT, catalase; GFP, green fluorescent protein; WT, wild type; kb, kilobase pair.

TABLE I
H. polymorpha strains and plasmids

Strains/plasmids	Relevant properties	Source or Ref.
H. polymorpha strains		
CBS 4732	Wild type (WT)	
WT::P_{AOX}*GFP.SKI*	CBS 4732 with integration of plasmid pHIPZ4-*GFP.SKI* at the P_{AOX} locus	This study
WT::P_{AOX}*GFP.LARF*	CBS 4732 with integration of plasmid pHIPZ4-*GFP LARF* at the P_{AOX} locus	This study
Δ*pex1*	*PEX1* deletion strain, *leu1.1*	40
Δ*pex1*::P_{AOX}*PEX5*^{mc}	Δ*pex1* with multicopy integration of plasmid pHIPX4-*PEX5* at the P_{AOX} locus	This study
Δ*pex3*	*PEX3* deletion strain, *leu1.1*	41
Δ*pex3*::P_{AOX}*PEX5*^{mc}	Δ*pex3* with multicopy integration of plasmid pHIPX4-*PEX5* at the P_{AOX} locus	This study
Δ*pex6*	*PEX6* deletion strain, *leu1.1*	40
Δ*pex6*::P_{AOX}*PEX5*^{mc}	Δ*pex6* with multicopy integration of plasmid pHIPX4-*PEX5* at the P_{AOX} locus	This study
Pex10–1	*pex10–1* mutant, *leu1.1*	42
Pex10–1::P_{AOX}*PEX5*^{mc}	*pex10–1* with multicopy integration of plasmid pHIPX4-*PEX5* at the P_{AOX} locus	This study
Δ*pex14*	*PEX14* deletion strain, *leu1.1*	24
Δ*pex14*::P_{AOX}*PEX5*^{2c}	Δ*pex14* with two-copy integration of plasmid pHIPX4-*PEX5* at the P_{AOX} locus	This study
Δ*pex14*::P_{AOX}*PEX5*^{mc}	Δ*pex14* with multicopy integration of plasmid pHIPX4-*PEX5* at the P_{AOX} locus	This study
Δ*pex14*::P_{AOX}*PEX5*^{mc}::P_{AOX}*GFP.SKI*	Δ*pex14*::P_{AOX}*PEX5*^{mc} with integration of plasmid pHIPZ4-*GFP.SKI* at the P_{AOX} locus	This study
Δ*pex14*::P_{AOX}*PEX5*^{mc}::P_{AOX}*GFP.LARF*	Δ*pex14*::P_{AOX}*PEX5*^{mc} with integration of plasmid pHIPZ4-*GFP.LARF* at the P_{AOX} locus	This study
Plasmids		
pBluescript II KS$^+$		Stratagene, La Jolla, CA
pPICZa		Invitrogen, Groningen, the Netherlands
pFEM39	pBluescript II KS$^+$ containing the zeocine selection cassette from pPICZa	This study
pEGFP-C1		Clontech, Palo Alto, CA
pHIPX4		43
pHIPX4-*PEX5*	pHIPX4 containing the PEX5 gene under control of the alcohol oxidase promoter (P_{AOX})	17
pHIPX4-*URA3-PEX5*	Plasmid pHIPX4-P_{AOX}*PEX5* containing the *URA3* gene	This study
pHIPZ4	pBluescript II KS$^+$-derived vector containing the alcohol oxidase promoter region (P_{AOX}), the amino oxidase terminator region (T_{AMO}), and the Zeocine resistance (Zeo^r) marker	This study
pHIPZ4-*GFP.SKI*	pHIPZ4 containing the *GFP* gene fused to the tripeptide SKI under control of the alcohol oxidase promoter (P_{AOX})	This study
pHIPZ4-*GFP.LARF*	pHIPZ4 containing the *GFP* gene fused to the tetrapeptide LARF under control of the alcohol oxidase promoter (P_{AOX})	This study

morpha Δ*pex4* cells. Remarkably, in these cells a significant portion of Pex5p accumulated at the inner surface of the peroxisomal membrane (29). A likely explanation for these observations is that the recycling of Pex5p is hampered in the absence of Pex4p thus making the import of newly synthesized matrix proteins dependent on newly formed Pex5p.

In this paper we show that, like in Δ*pex4* cells, overexpression of *PEX5* also significantly stimulates PTS1-protein import in Δ*pex14* cells. In Δ*pex14* cells overexpressing *PEX5* significant amounts of Pex5p accumulate at the outer surface of the peroxisomal membrane, which suggested that (under these conditions) Pex14p is not necessary for the association of Pex5p with the peroxisomal membrane.

MATERIALS AND METHODS

Organisms and Growth Conditions—The *H. polymorpha* strains used in this study are listed in Table I. *H. polymorpha* was grown at 37 °C in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (w/v) glucose or in mineral medium (30) supplemented with 0.5% (w/v) glucose or 0.5% (v/v) methanol as carbon source and 0.25% (w/v) ammonium sulfate or methylamine as nitrogen source.

Construction of Pex Mutants Overexpressing PEX5—H. polymorpha Δ*pex1*, Δ*pex3*, Δ*pex6*, *pex10.1,* and Δ*pex14* mutants were transformed with *Stu*I-linearized plasmid pHIPX4-*PEX5* (Table I) using electroporation (31). Site-directed integration of the plasmids at the P_{AOX} locus was confirmed by Southern blot analysis. Strains containing multiple copies of the pHIPX4 derivatives were selected as reported earlier (29).

Construction of pHIPZ4—First the zeocine selection cassette was taken from pPICZa (Table I) as a 1.1-kb *Mlu*I (Klenow fill-in of sticky ends)-*Bam*HI fragment and inserted into *Asp*718 (Klenow fill-in of sticky ends)-*Bam*HI-digested pBluescript II KS$^+$ (Table I), resulting in pFEM39. Subsequently, a 2.0-kb *Not*I-*Bgl*II fragment, containing the alcohol oxidase promoter region (P_{AOX}) and amine oxidase terminator region (T_{AMO}), was taken from pHIPX4 (Table I) and inserted into *Not*I-*Bam*HI0-digested pFEM39, resulting in pHIPZ4.

Construction of Strains Producing PTS1-containing GFP Proteins—The chimeric gene encoding GFP fused to the targeting signal of AO (LARF-COOH) was constructed by polymerase chain reaction-mediated amplification of the *GFP* gene of pEGFP-C1 (Table I) using the following oligonucleotides (oligonucleotides were obtained from Life Technologies, Inc.): upstream 5′-AGA AAG CTT ATG GTG AGC AAG GGC-3′ (*Hin*dIII site underlined) and downstream 5′-CCC GTC GAC TTA *GAA TCT GGC CAG* CTT GTA CAG CTC GTC-3′ (*Sal*I site underlined and the nucleotides that code for the tetrapeptide LARF are depicted in italics). For the construction of the gene encoding GFP fused to the targeting signal of CAT (SKI-COOH) the following primers were used: upstream 5′-CCC GGA TCC ATG GTG AGC AAG GGC GAG-3′ (*Bam*HI site underlined) and downstream 5′-CCC GTC GAC TTA *GAT CTT CGA* CTT GTA CAG CTC GTC-3′ (*Sal*I site underlined and the nucleotides that code for the tripeptide SKI are depicted in italics).

The polymerase chain reaction products were ligated as 0.8-kb *Bam*HI-*Sal*I or *Hin*dIII-*Sal*I fragments in pHIPZ4, digested with *Bam*HI-*Sal*I or *Hin*dIII-*Sal*I. The resulting plasmids, pHIPZ4-*GFP.SKI* and pHIPZ4-*GFP.LARF*, were linearized with *Sph*I and integrated at the P_{AOX} locus of *H. polymorpha* WT and Δ*pex14*::P_{AOX}*PEX5*^{mc}. Transformants were selected on YPD plates containing 0.1 mg/ml zeocine

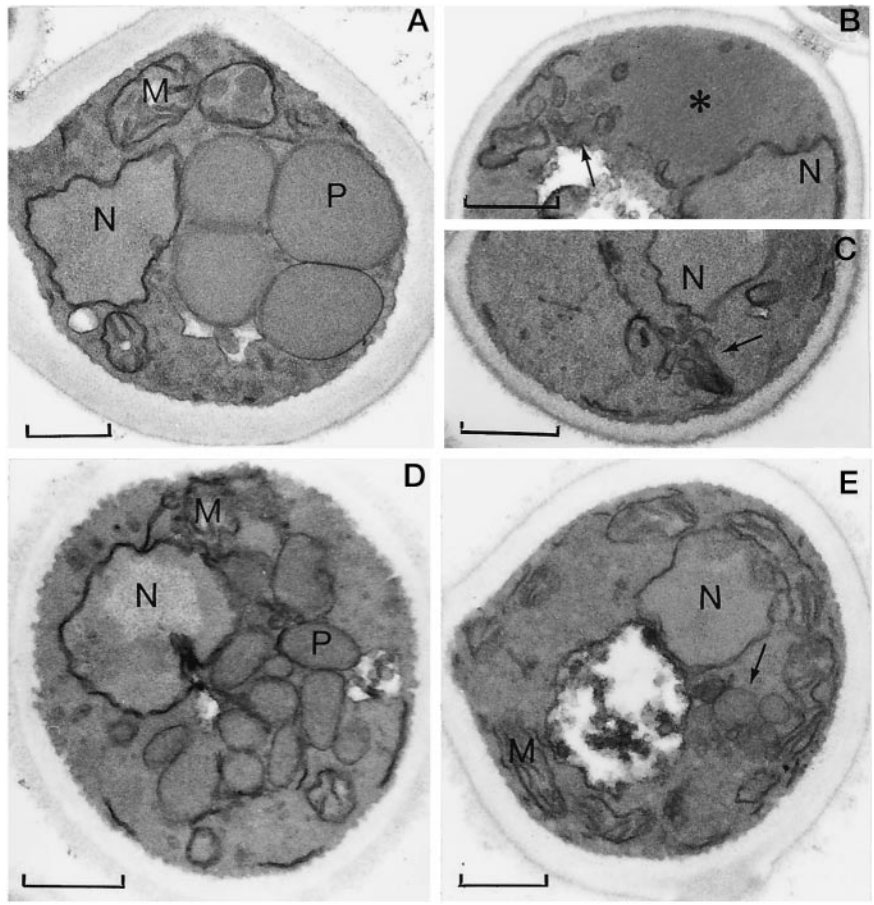

FIG. 1. **Overall morphology of methanol-induced *H. polymorpha* cells to show the re-introduction of peroxisomes in Δ*pex14* cells, which overproduce Pex5p.** In methanol-grown WT control cells normal peroxisomes are present (*A*). Peroxisome development is not restored in *pex10−1*::P_{AOX}*PEX5* cells incubated for 24 h in methanol containing media (*B*) (*arrow,* peroxisomal remnants; *, AO aggregate). Methanol-induced Δ*pex14* cells contain peroxisomal remnants (*C*) (*arrow*, peroxisomal remnants). However, in identically grown Δ*pex14*::P_{AOX}*PEX5*^{mc} cells several peroxisomes are present (*D*). Also, when lower Pex5p levels are obtained in Δ*pex14*, *i.e.* in Δ*pex14*::P_{AOX}*PEX5*^{2c} grown for 16 h on glycerol, several small peroxisomes (*arrow*) are observed in the cells (*E*). Electron micrographs are taken of $KMnO_4$-fixed cells, poststained with uranyl acetate. The abbreviations used are as follows: *M,* mitochondrion; *N,* nucleus; *P,* peroxisome; *V,* vacuole. The marker represents 0.5 μm.

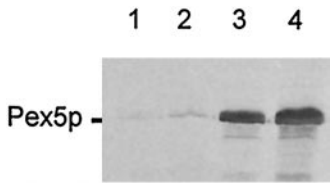

FIG. 2. **Western blot showing the level of Pex5p in crude extracts of methanol-grown WT (*lane 1*), methanol-induced Δ*pex14* (*lane 2*), Δ*pex14*::P_{AOX}*PEX5*^{2c} (*lane 3*), and Δ*pex14*::P_{AOX}*PEX5*^{mc} (*lane 4*) using α-Pex5p antibodies.** Compared with WT and Δ*pex14* extracts, Δ*pex14*::P_{AOX}*PEX5*^{2c} and Δ*pex14*::P_{AOX}*PEX5*^{mc} extracts contained enhanced amounts of Pex5p.

(Invitrogen, Groningen, The Netherlands). Site-directed integration of the plasmid at the P_{AOX} locus was confirmed by Southern blot analysis.

Biochemical Methods—Alcohol oxidase (32), catalase (33), and cytochrome *c* oxidase (34) were assayed as described. Protein concentrations were determined using the Bio-Rad Protein Assay system (Bio-Rad) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out as described (35), and gels were subjected to Western blotting (36). Nitrocellulose blots were decorated using specific polyclonal antibodies against various *H. polymorpha* proteins. Cell fractionation (37), floatation centrifugation (38), and proteinase K protection assays (29) were performed as described before.

Electron Microscopy—Cells were fixed and prepared for electron microscopy as described previously (39). Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against various proteins and gold-conjugated goat anti-rabbit antibodies (39).

RESULTS

Peroxisome Formation Is Restored in Δpex14 Cells Overproducing Pex5p—Previously, we observed that overexpression of *PEX5* restored the formation of peroxisomes in *H. polymorpha* Δ*pex4* cells (29). To determine whether this phenomenon also occurs in other *H. polymorpha pex* mutants, a *PEX5* overexpression cassette (P_{AOX}*PEX5*) was integrated in two or multiple (3–5) copies into the genomes of Δ*pex1,* Δ*pex3,* Δ*pex6, pex10−1,* and Δ*pex14* (Table I). Cells of these strains were incubated for 24 h in methanol-containing media to induce the P_{AOX} and the major peroxisomal PTS1 proteins AO and DHAS. None of the strains showed significant growth on methanol indicating that full restoration of peroxisome formation did not occur in any of the strains. We subsequently examined thin sections of $KMnO_4$-fixed cells from these cultures for the presence of peroxisomal structures. Methanol-grown WT cells, used as a control, characteristically contain several large peroxisomes (Fig. 1*A*). In Δ*pex1,* Δ*pex3,* Δ*pex6,* and *pex10−1* cells overexpressing *PEX5*, such organelles were invariably absent (Fig. 1*B*, *pex10−1*; others not shown). However, in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells the presence of several peroxisomes was evident. The size of these organelles was slightly reduced compared with those present in WT control cells (Fig. 1*D*).

In order to compare the Pex5p levels in WT, Δ*pex14* and Δ*pex14* overproducing Pex5p Western blotting experiments were performed. These experiments revealed that methanol-induced WT and Δ*pex14* cells contain similar levels of Pex5p. The Pex5p level was, however, significantly increased in strains containing two or multiple copies of the P_{AOX}*PEX5* overexpression cassette (Fig. 2). Densitometric scanning of blots prepared from crude extracts of methanol-grown WT cells and Δ*pex14*::P_{AOX} *PEX5*^{mc} cells induced for 24 h on methanol-containing media revealed that in Δ*pex14*::P_{AOX}*PEX5*^{mc} the amount of Pex5p was approximately 100-fold higher than in WT control cells. Much lower overproduction of Pex5p (*i.e.* a 20-fold increase compared with WT cells) was obtained in a Δ*pex14* strain containing only two copies of the P_{AOX}*PEX5* cassette (Δ*pex14*::P_{AOX}*PEX5*^{2c}) upon growth for 16 h on glycerol-containing medium. Examination of ultrathin sections pre-

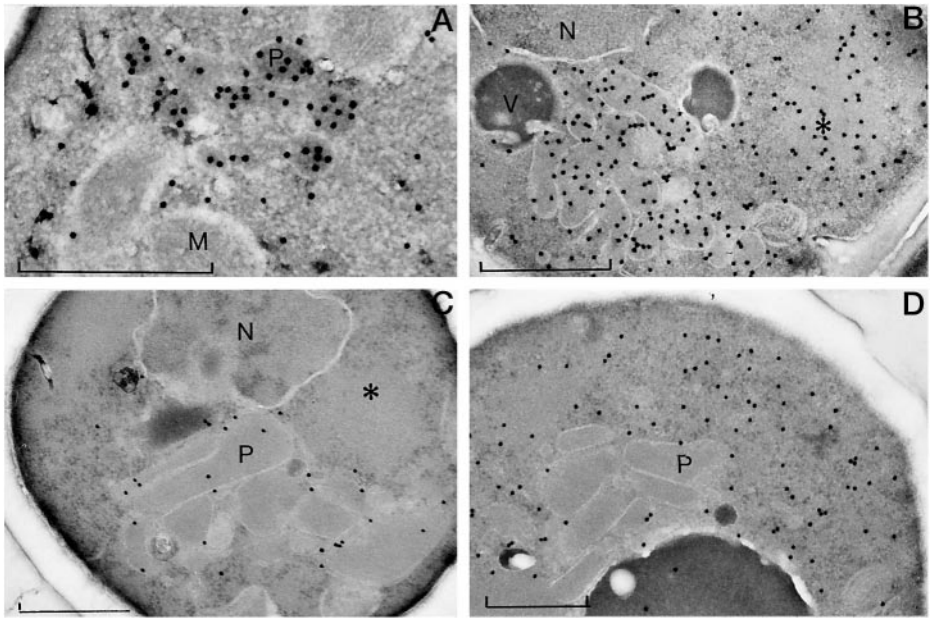

FIG. 3. **Immunocytochemical demonstration of peroxisomal proteins in glycerol-grown Δ*pex14*::P_{AOX}*PEX5*^{2c} cells (*A*) or methanol-induced Δ*pex14*::P_{AOX}*PEX5*^{mc} (*B–D*).** By using α-AO antisera the small peroxisomes present in glycerol-grown Δ*pex14*::P_{AOX}*PEX5*^{2c} cells are labeled. Part of the AO protein is, however, mislocated in the cytosol (*A*). A similar labeling pattern is observed using α-DHAS antisera in methanol-induced induced Δ*pex14*::P_{AOX}*PEX5*^{mc} (*B*) (*, AO aggregate). The peroxisomal membrane protein Pex3p is properly inserted into the membrane of these peroxisomes as was evident upon labeling using α-Pex3p antibodies (*C*) (*, AO aggregate). Unexpectedly, CAT protein was confined to the cytosol and absent in the peroxisomes present in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells (α-CAT; *D*) glutaraldehyde-fixation. The abbreviations used are as follows: *M,* mitochondrion; *N,* nucleus; *P,* peroxisome; *V,* vacuole. The marker represents 0.5 μm.

pared from $KMnO_4$-fixed cells of this culture revealed that also a 20-fold increase in Pex5p levels resulted in the formation of small peroxisomes in *H. polymorpha* Δ*pex14* (Fig. 1*E*).

PTS1 Protein Import Is Stimulated by Pex5p Overproduction in Partially Restored Δ*pex14 Cells*—In methanol-induced *H. polymorpha* Δ*pex14* cells normal peroxisomes are absent. Instead these cells contain peroxisomal membrane remnants that contain a very minor portion of AO and DHAS, whereas the bulk of these proteins is mislocated in the cytosol (24). Immunocytochemical analysis of glycerol-grown Δ*pex14*::P_{AOX}*PEX5*^{2c} cells revealed that the small peroxisomes that are formed in these cells contain the PTS1 proteins AO (Fig. 3*A*) and DHAS (not shown). Similarly, the larger organelles present in methanol-induced Δ*pex14*::P_{AOX}*PEX5*^{mc} cells are characterized by significant amounts of AO (not shown) and DHAS protein (Fig. 3*B*). Judged from the distribution of the labeling, a portion of these matrix proteins was still mislocated in the cytosol. By using antibodies against Pex3p, a peroxisomal membrane protein, labeling was obtained at the membrane of these organelles (Fig. 3*C*), indicating that they indeed represent peroxisomes. Remarkably, import of a third PTS1 protein, catalase (CAT), was not significantly stimulated by Pex5p overproduction (Fig. 3*D*). As expected, the location of the PTS2 protein amine oxidase remained cytosolic in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells (data not shown).

The immunocytochemical data were confirmed biochemically by cell fractionation experiments. To this end post-nuclear supernatants, prepared from methanol-grown WT cells or methanol-induced Δ*pex14* and Δ*pex14*::P_{AOX}*PEX5*^{mc} cells, were subjected to sucrose density centrifugation (Fig. 4). In gradients prepared from WT cells, peroxisomes migrate to a high density corresponding to 54% sucrose (*fraction 6*, Fig. 4*A*), indicated by a distinct peak of AO and CAT activity. Also the peroxisomal membrane marker Pex3p is present in these fractions, as evident from Western blots prepared from the gradient fractions (*inset* in Fig. 4*A*). The AO and CAT activities detected in the top fractions of the gradient most likely are due to leakage of these proteins as a result of the fractionation procedure (37). In the gradient of Δ*pex14* cells (Fig. 4*B*) AO, CAT, and Pex3p are absent at high densities. Instead, AO and CAT activities are present at the top of the gradient, indicative for a cytosolic location. Pex3p migrated to a density corresponding to 37% sucrose (*fraction 17*, Western blot included in Fig. 4*B*). Previously, evidence was provided using immunocytochemistry that the peroxisomal membrane remnants, present in Δ*pex14* cells, contained Pex3p and very small amounts of AO and DHAS (24). Hence, these structures most likely migrated to a density corresponding to 37% sucrose (*fraction 17*) of the gradient prepared from Δ*pex14* cells. Gradients of methanol-induced Δ*pex14*::P_{AOX}*PEX5*^{mc} cells again displayed a prominent peak of AO activity at 54% sucrose (*fraction 7*, Fig. 4*C*); also Pex3p was localized at this position (Western blot included in Fig. 4*C*). However, significant amounts of AO activity, exceeding those of WT controls, were also detected in the top fractions of this gradient (fraction 18–25) most likely representing the cytosolic portion of the AO protein demonstrated by immunocytochemistry (Fig. 3*A*). In contrast, CAT activity (Fig. 4*C*) and CAT protein, as determined by Western blotting (data not shown), was only detected in the top fractions of this gradient, thus confirming the immunocytochemical data that the import of CAT was not stimulated in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells.

To obtain additional evidence that the AO activity peak detected at 54% sucrose in the Δ*pex14*::P_{AOX}*PEX5*^{mc} gradient indeed represented membrane-bound AO protein and was not due to the presence of dense AO protein aggregates and/or crystalloids, these fractions were subjected to floatation centrifugation. As shown in Fig. 5, both AO activity and the peroxisomal membrane protein Pex3p floated to lower densities and comigrated in a distinct protein peak at approximately 50% sucrose, indicating that AO is indeed membrane-bound.

Restoration of PTS1 Import Is Not Related to Variations in the PTS1—Overproduction of Pex5p in Δ*pex14* cells significantly stimulated import of the PTS1 proteins AO and DHAS

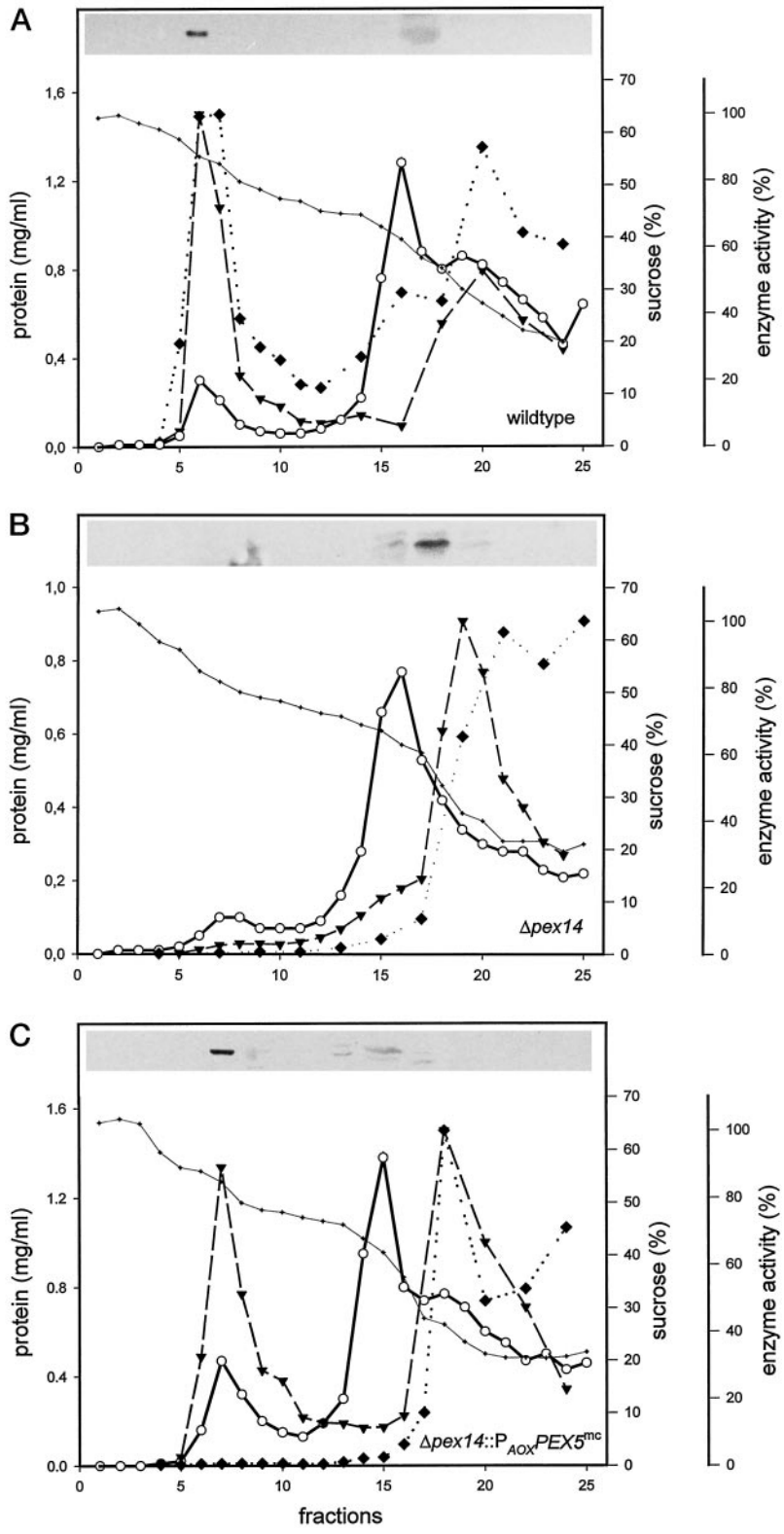

FIG. 4. **Sucrose gradients, prepared from post-nuclear supernatants obtained from homogenized, methanol-induced *H. polymorpha* WT cells (*A*), Δ*pex14* cells (*B*), and Δ*pex14*::P$_{AOX}$-*PEX5*^{mc} cells (*C*), showing the distribution of the activities of the peroxisomal enzymes AO (▼) and CAT (♦) in these gradients.** In the gradient of the Δ*pex14* cells, AO and CAT activities are mainly present at the top of the gradient, indicating that they are soluble; Pex3p accumulates in fraction 17 and therefore is most probably bound to peroxisomal remnants. In the gradient of Δ*pex14*::P$_{AOX}$*PEX5*^{mc} cells a considerable portion of AO, but not CAT activity, is again located at fraction 7, where normal WT peroxisomes accumulate (*A*); also Pex3p has again migrated to these fractions (compare Western blots of *A*–*C*). For clarity, the pattern of the mitochondrial enzyme distribution is not indicated in these graphs; however, in all three gradients the mitochondrial peak fraction, judged from cytochrome *c* oxidase activities, coincided with the protein peaks at fraction 15. Sucrose concentrations (+) are expressed as percentages (w/w,) the protein concentrations (○) as mg/ml. The specific activities of AO and CAT are expressed as percentages of the activity in the peak fractions, which were arbitrarily set at 100. The Western blots at the *top* of each panel show the distribution of Pex3p in this gradient, as determined in the even fractions of the gradients. Equal portions of the fractions were used per lane.

but not of CAT. Possibly, this may be related to differences in affinities of *H. polymorpha* Pex5p for the PTS1 sequences of these proteins, *i.e.* LARF-COOH in case of AO, NKL-COOH in case of DHAS, and SKI-COOH in case of CAT (8, 9). To analyze this possibility, we constructed chimeric genes encoding green fluorescent protein (GFP) fused to the respective targeting signals of AO and CAT (GFP.LARF and GFP.SKI, respectively). These genes were introduced in Δ*pex14*::*P*$_{AOX}$*PEX5*^{mc} and a WT control strain, and the location of their translation products was determined. In *H. polymorpha* WT cells the two GFP fusion proteins were efficiently targeted to the peroxisomes, as indicated by the punctate patterns observed by fluorescence microscopy (data not shown) and immunocytochemistry, using specific α-GFP antibodies (Fig. 6*A*, GFP.LARF and GFP.SKI, not shown). However, in Δ*pex14*::*P*$_{AOX}$*PEX5*^{mc} cells, producing either GFP.LARF or GFP.SKI, a diffuse fluorescence pattern was observed, indicative for a cytosolic location (data not shown). To examine whether minor amounts of the fusion proteins may

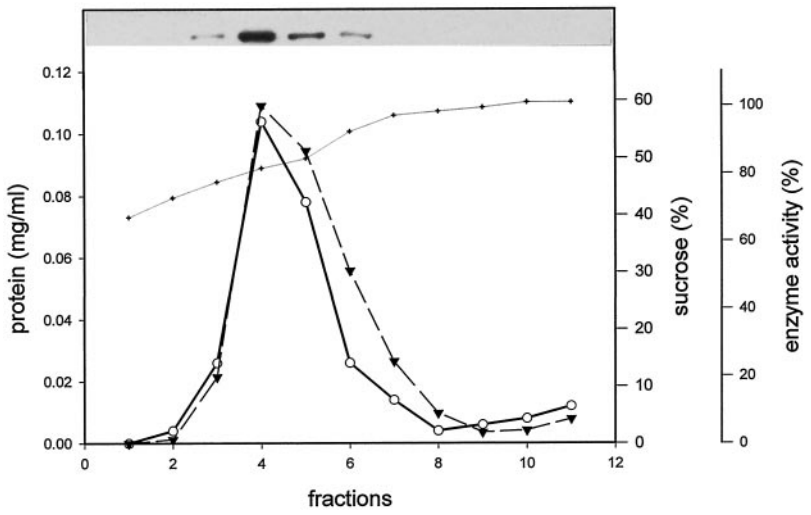

FIG. 5. **Floatation gradient of the peroxisomal peak fraction of a sucrose gradient prepared from Δ*pex14*::P$_{AOX}$*PEX5*^{mc} cells (*fraction 7*, Fig. 4*C*).** The peroxisomal AO activity peak coincides with the protein peak in *fraction 4* at 50% sucrose (+). In this fraction also Pex3p is present, as is evident from the Western blot. The protein concentration (○) is expressed as mg/ml and the AO activity (▼) as a percentage of the activity present in the peak fraction, which was arbitrarily set at 100. The Western blot shows the distribution of Pex3p in the fractions of the gradient, using α-Pex3p antibodies. Equal portions of the fractions were loaded per lane.

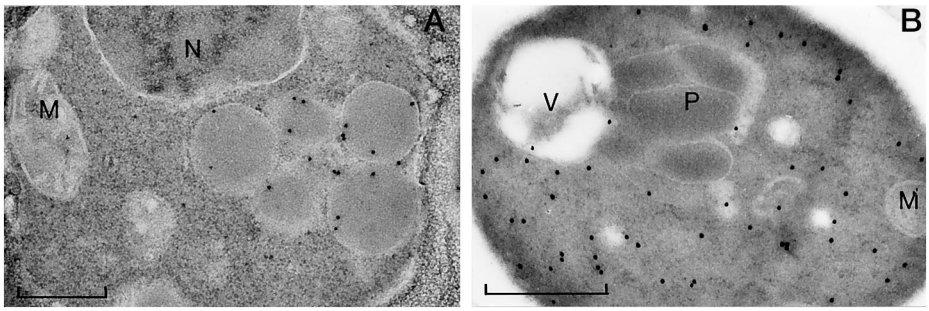

FIG. 6. **Immunocytochemical localization of GFP in WT::P$_{AOX}$*GFP*.** *LARF* (*A*) and Δ*pex14*::P$_{AOX}$*PEX5*^{mc}::P$_{AOX}$*GFP.LARF* cells (*B*). Using α-GFP antibodies, labeling is observed in the matrix of the peroxisomes in WT::P$_{AOX}$*GFP.LARF* cells. The protein is predominantly present at the periphery of the organelle, due to the presence of an AO crystalloid in the organellar matrix (*A*). In Δ*pex14*::P$_{AOX}$*PEX5*^{mc} cells producing GFP.LARF, labeling is restricted to the cytosol (*B*). The abbreviations used are as follows: *M,* mitochondrion; *N,* nucleus; *P,* peroxisome; *V,* vacuole. The marker represents 0.5 μm.

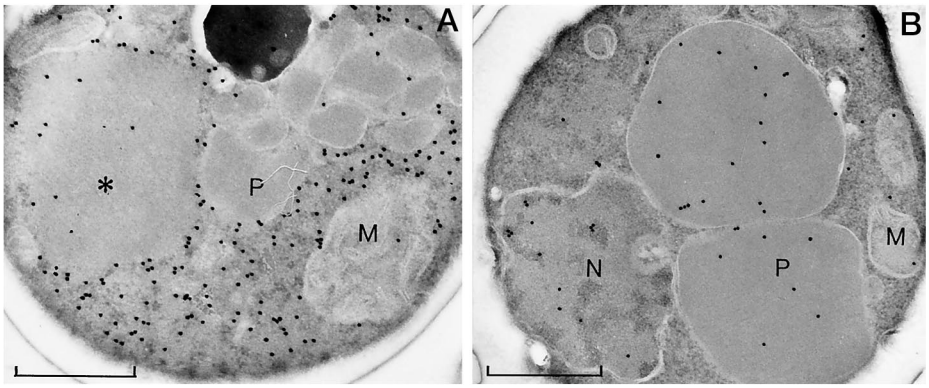

FIG. 7. **Immunocytochemical localization of Pex5p on ultrathin sections of methanol-induced Δ*pex14*::P$_{AOX}$*PEX5*^{mc} cells (*A*) and WT control cells (*B*), using α-Pex5p antibodies.** In Δ*pex14*::P$_{AOX}$*PEX5*^{mc} cells the bulk of the labeling is localized in the cytosol with few particles associated with the peroxisomal membrane, whereas in WT cells labeling is observed in the cytosol (including the nucleus) and the peroxisomal matrix (*, AO crystalloid). The abbreviations used are as follows: *M,* mitochondrion; *N,* nucleus; *P,* peroxisome; *V,* vacuole. The marker represents 0.5 μm.

be imported into peroxisomes, their location was also determined immunocytochemically. The labeling patterns, using α-GFP antibodies, revealed that both proteins were confined to the cytosol (Fig. 6*B*, GFP.LARF and GFP.SKI, not shown).

Pex5p Accumulates in the Cytosol and at the Outer Surface of the Peroxisomal Membrane in Δpex14::P$_{AOX}$PEX5^{mc} Cells—In WT *H. polymorpha* Pex5p has a dual location and is present both in the cytosol and the peroxisomal matrix (Fig. 7*B*); a similar location is found in WT cells overproducing Pex5p (not shown; see Ref. 17). In immunocytochemical experiments using ultrathin sections of methanol-induced Δ*pex14*::P$_{AOX}$*PEX5*^{mc} cells, the bulk of Pex5p labeling was found at the cytosol. In addition, labeling was observed at the peroxisomal membrane but never on the organellar matrix (Fig. 7*A*). In sucrose gradients of a post-nuclear supernatant prepared from homogenized, methanol-induced Δ*pex14*::P$_{AOX}$*PEX5*^{mc} cells, Pex5p sedimented at two specific locations. Pex5p cosedimented with peroxisomal marker proteins at 54% sucrose (fraction 7) and was also detected at top of the gradient where cytosolic proteins are located (*fractions 18–23*; Fig. 8*A*). Characteristically, the cytosolic Pex5p was predominantly detected at lower molecular weight due to proteolytic degradation. Taken together, these data indicate that

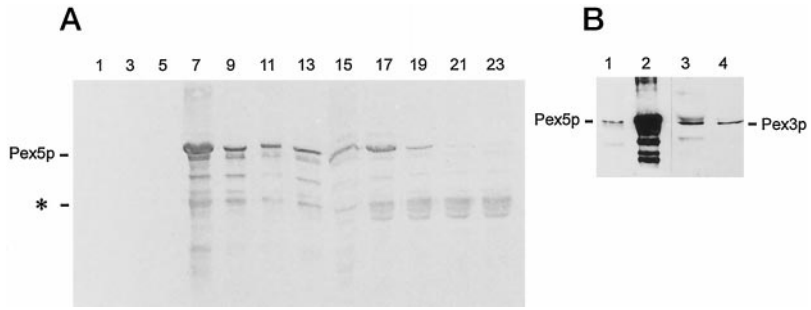

FIG. 8. *A,* Western blot showing the distribution of Pex5p in the odd fractions of a sucrose gradient prepared from homogenized methanol-induced Δ*pex14*::P_{AOX}*PEX5*^{mc} cells (compare Fig. 4*C*) using α-Pex5p antibodies. A prominent band is observed at the position were WT peroxisomes are located (*fraction 7*). The soluble portion of Pex5p protein has most likely been subject to proteolytic degradation, as is suggested by the strong protein bands at 30 kDa in fractions 17–23 (indicated by *). *B,* Western blots prepared from purified peroxisomes, using α-Pex5p (*lanes 1* and *2*) and α-Pex3p (*lanes 3* and *4*) antibodies. Compared with WT peroxisomes (*lane 1*), peroxisomes from Δ*pex14*::P_{AOX}*PEX5*^{mc} cells contain enhanced amounts of Pex5p (*lane 2*). The amount of Pex3p is somewhat higher than in WT organelles (*lane 3*) compared with peroxisomes from Δ*pex14*::P_{AOX}*PEX5*^{mc} cells (*lane 4*). Peroxisomes were purified by sucrose density centrifugation. Equal amounts of protein were loaded per lane.

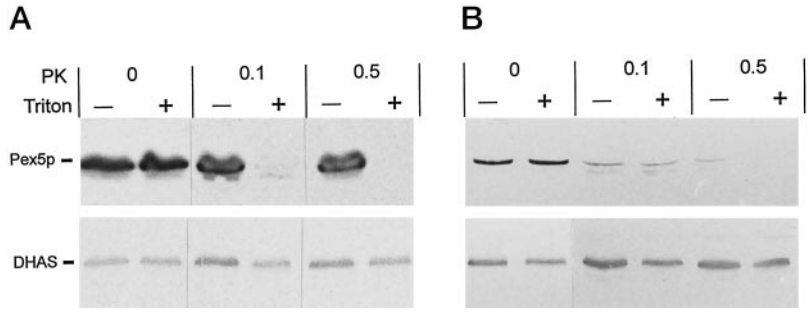

FIG. 9. **Protease protection analysis using purified peroxisomes from methanol-induced WT (*A*) and Δ*pex14*::P_{AOX}*PEX5*^{mc} cells (*B*).** Fractions were incubated in the presence of the indicated amounts (in mg/ml) of proteinase K (*PK*) with (+) or without (−) 0.1% Triton X-100 for 30 min at 0 °C. The Western blots were decorated with antibodies against Pex5p and DHAS. Pex5p in WT peroxisomes is only degraded in the presence of Triton X-100, whereas in Δ*pex14*::P_{AOX}*PEX5*^{mc} peroxisomes Pex5p is rapidly degraded independently from detergent. The matrix protein DHAS, used as control, is relatively stable and is only partially degraded in the presence of proteinase K and Triton X-100, as is indicated by the weaker protein band observed in the samples containing both proteinase K and Triton X-100 compared with the samples lacking proteinase K (also AO is relatively stable, whereas CAT, which is more sensitive to proteases, cannot be used in this experiment). Equal portions of proteins were loaded in each experiment, except for the detection of Pex5p in WT peroxisomes, in which the protein concentration was 40-fold enhanced to facilitate Pex5p detection.

Pex5p is present in both cytosolic and peroxisomal fractions in gradients prepared from Δ*pex14*::P_{AOX}*PEX5*^{mc} cells.

Western blotting experiments revealed that the amounts of Pex5p in peroxisomal peak fractions of Δ*pex14*::P_{AOX}*PEX5*^{mc} gradients were strongly enhanced (>40-fold) compared with WT controls (Fig. 8*B*); in these samples the levels of Pex3p, used as a control, were comparable (Fig. 8*B*).

In order to determine the precise location of Pex5p in peroxisomes of Δ*pex14*::P_{AOX}*PEX5*^{mc} cells, a protease protection assay was performed. Incubation of purified peroxisomes with proteinase K resulted in the degradation of most of the Pex5p, independent of permeabilization by Triton X-100 (Fig. 9*B*). Only a very minor amount of Pex5p was protected against proteolytic degradation using 0.5 mg/ml proteinase K. In control experiments, using purified peroxisomes from WT cells, all Pex5p was protected against proteinase K; degradation of the protein was only observed when the organelles were permeabilized. This confirms earlier findings that in *H. polymorpha* WT cells the peroxisomal fraction of Pex5p is located inside the organellar matrix (17).

Our results indicate that in Δ*pex14*::P_{AOX}*PEX5*^{mc} the additional Pex5p that is associated with peroxisomes (*i.e.* a 40-fold increase compared with WT) is predominantly associated with the outer surface of the peroxisomes, whereas a minor portion is present inside the organelles. This amount may be comparable to that observed in peroxisomes from WT cells, where all peroxisome-associated Pex5p is located inside the organelle.

DISCUSSION

Pex14p is a peroxisomal membrane protein and is essential for peroxisome biogenesis (24–26, 44–46). The finding that Pex14p is able to bind Pex5p and Pex7p, the specific receptors for the PTS1 and PTS2, respectively (25), has led to the suggestion that Pex14p is a central component of the peroxisomal protein translocation machinery, most likely mediating the membrane docking event of these receptors. A similar function has been postulated for Pex13p; moreover, Pex13p and Pex14p have been shown to physically interact (21–23, 47). Pex13p and Pex14p are probably part of a protein complex, which is characterized by a rather fixed stoichiometry of its constituting components for normal functioning (24, 38). Recently, a potential third component of this complex, Pex17p, has been identified (27).

Pex14p Is Not Essential for Accumulation of Pex5p at the Organellar Surface—We have shown that *H. polymorpha* cells, lacking Pex14p, could import specific PTS1 proteins under conditions that Pex5p was overproduced. A dose-response correlation was observed between the level of Pex5p produced and the amount of PTS1 proteins imported. A plausible explanation for restoration of import is to assume that the import of these proteins proceeds via a series of consecutive steps (comparable to matrix protein import in mitochondria for example), one of which involves the function of Pex14p (Fig. 10). Accordingly, it can be envisaged that excess amounts of Pex5p can compensate for the absence of a single component of this machinery, *i.e.*

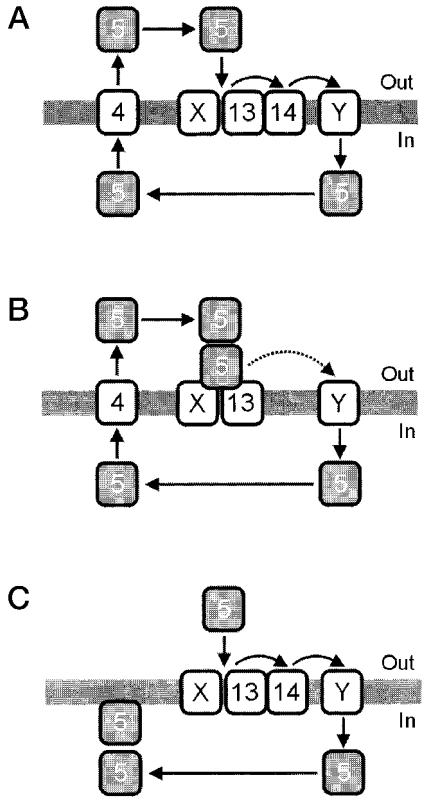

FIG. 10. **Schematic representation of hypothetical pathways of Pex5p in PTS1-protein import in *H. polymorpha* WT cells (*A*), Δ*pex14*::P_{AOX}*PEX5*^{mc} cells (*B*), and Δ*pex4*::P_{AOX}*PEX5* cells (*C*).** *A,* upon binding of a newly synthesized PTS1 protein to cytosolic Pex5p, Pex5p associates with a Pex5p docking complex on the peroxisomal membrane, which are thought to contain Pex13p, and additional components (*X*) that have not been identified yet. Following docking Pex5p is directed to a putative translocation site (*Y*) via a series of consecutive steps, one of which involves the function of Pex14p. After translocation of the Pex5p-PTS1 protein complex across the peroxisomal membrane the PTS1 protein is released, whereas Pex5p is recycled back to the cytosol, a process that involves Pex4p. *B,* in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells transfer of Pex5p from the docking site to the translocation site (*Y*) is affected due to the absence of Pex14p. As a result Pex5p accumulates at the initial docking site (*X*/Pex13p). However, increased concentrations of cytosolic Pex5p compensate for the absence of Pex14p, resulting in PTS1 protein import. *C,* in Δ*pex4* cells recycling of Pex5p from the matrix to the cytosol is blocked (29). Also in these cells the PTS1 protein import defect can be restored by Pex5p overproduction. However, this results in accumulation of Pex5p at the inner surface of the peroxisomal membrane.

Pex14p. The accompanying phenomenon, namely the accumulation of Pex5p at the peroxisomal membrane, could then be understood by assuming that Pex14p is in fact not the initial docking site of the Pex5p-cargo complex but functions at a later stage in the import pathway where it is essential for the efficiency of this process. Consequently, the absence of Pex14p would then strongly slow down import and could rise to the accumulation of Pex5p, blocked at the early steps of translocation (Fig. 10). It should be stressed that in WT *H. polymorpha* Pex5p was never found to be accumulated to the outer surface of the organelle (Fig. 7*B*; 17), even when *PEX5* was overexpressed (29). However, in other organisms membrane-bound Pex5p has been observed (21, 23, 48). In particular, in mammalian cells enhanced amounts of Pex5p were bound to the peroxisomal surface under conditions that inhibited protein translocation (*e.g.* upon ATP-depletion, low temperature; Ref. 50); a similar observation was made in mutant cell lines blocked in matrix import due to mutations in *PEX2* or *PEX12* (49). The mechanisms, underlying these phenomena, could in fact be comparable to those made in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells, namely that, dependent on the nature of the block in import, Pex5p can accumulate at the peroxisomal membrane. Remarkably, the location of Pex5p in peroxisomes of Δ*pex14*::P_{AOX}*PEX5*^{mc} cells was opposite that in Δ*pex4* cells, overproducing Pex5p, in that the latter cells Pex5p had accumulated at the luminal site of the peroxisomal membrane (29) (Fig. 10).

If we assume that Pex14p is not involved in the initial recruiting of Pex5p from the cytosol, this process should be fulfilled by other peroxisomal membrane proteins to explain the import in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells. A possible candidate is Pex13p, but the involvement of additional proteins cannot be ruled out. An example of this could be a recently identified 140-kDa protein in peroxisomal membranes of rat liver which binds human Pex5p in ligand blots (45). However, this protein was not further analyzed.

Pex5p Overproduction in Δpex14 Cells Suppresses the Import Defect of Specific PTS1 Proteins—The finding that the peroxisomal remnants in *H. polymorpha* Δ*pex14* cells do contain trace amounts of AO protein implies that the PTS1 protein import defect in these cells is not 100% blocked (24). However, to our surprise the Pex5p-dependent import restoration in Δ*pex14* cells did not apply for all *H. polymorpha* PTS1 proteins and was only observed for AO and DHAS but not for CAT. Apparently, this difference is not related to variations in the PTS1 signal of these proteins since fusion proteins consisting of GFP fused to either the PTS1 of AO (LARF-COOH) or CAT (SKI-COOH) both were mislocalized to the cytosol of Δ*pex14*::P_{AOX}*PEX5*^{mc} cells.

As shown before, in *H. polymorpha* WT cells the presence of a PTS1 signal at the extreme carboxyl terminus is required and sufficient to direct a reporter protein to the peroxisomal matrix, a process that involves the function of the PTS1 receptor Pex5p (8, 9, 17). However, the differential import observed in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells (*e.g.* AO compared with GFP.LARF), overproducing Pex5p, suggests that for normal protein translocation additional factors are also involved. These factors may be related to intrinsic properties of the corresponding proteins (*e.g.* in case of AO and DHAS) and/or may even include yet unknown "accessory" proteins (*e.g.* in case of CAT). The first option, mentioned above, is not only hypothetical since evidence has been obtained that specific peroxisomal matrix proteins contain such information. A relevant example of this is that the removal of the PTS1 from *Pichia pastoris* AO did only result in a partial block in import of the truncated protein (50). Another indication is the finding that in two-hybrid assays the PTS1 protein carnitine acetyltransferase from *Saccharomyces cerevisiae* interacts with Pex5p, also when the PTS1 is removed (51).

However, the finding that CAT is not imported in Δ*pex14*::P_{AOX}*PEX5*^{mc} cells lends support to the notion that the PTS1 import machinery is not uniform for all PTS1 proteins. A comparable phenomenon has been observed in case of PTS2 protein import in *Yarrowia lipolytica*; in this organism Pex20p is specifically involved in the import and oligomerization of the PTS2 protein thiolase (52). Other examples include *Candida boidinii* Pmp47 which is specifically required for import and assembly of DHAS (53) and FAD, which is essential for efficient import of AO in peroxisomes of *H. polymorpha* (54). Above this, variations may exist with respect to the specific requirements of import, *e.g.* related to the folding/oligomerization properties of imported proteins (import as monomer or oligomer), the energy requirements, or the kinetics of import. However, further experiments are required to elucidate the details of the PTS1 protein import machinery.

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